American Journal of CLINICAL PATHOLOGY

TECHNICAL SUPPLEMENT

VOL. 3 JANUARY, 1939 No. 1

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PUBLISHED BI-MONTHLY BY THE WILLIAMS & WILKINS COMPANY

MOUNT ROYAL AND GUILFORD AVES., BALTIMORE, U. S. A.

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ANNOTATIONS, MINOR CONTRIBUTIONS, QUERIES

Under the above caption will be published from time to time comments, criticisms and suggestions on technical procedures; minor contributions such as laboratory aids and short cuts which are not considered sufficiently important to warrant a formal paper; and queries.

Obviously comments and criticisms should be signed; queries should be signed but names will be withheld on request; full credit will be given those who contribute laboratory aids, short cuts and the like.

An attempt will be made to obtain answers from authoritative sources to the queries submitted. It must be emphasized that the views expressed in this department are not the opinions of any official body.

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A RAPID BUT PRECISE GRAVIMETRIC METHOD FOR SERUM PROTEIN AND SERUM ALBUMIN*

EVALYN BARRETT

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A chance observation led to the development of this method. While making observations on the relation between the volume and weight of protein precipitates it was noted that protein precipitated by an acid alcohol solution of phosphotungstic acid and packed by centrifugilization shrinks when heated so that it can be removed en masse and without loss from the centrifuge tube. If this cone of precipitated protein is smeared as a film over the surface of a watch-glass it will reach a constant weight after fifteen minutes at 110 degrees Centigrade and is in such a state that it does not take up moisture from the air and can be weighed directly.

METHOD FOR TOTAL SERUM PROTEIN

1. Pipette 0.5 cc. serum† into a 15 cc. centrifuge tube.
2. Carefully layer on 95 per cent alcohol to 7 cc. and then add Tsuchiya's reagent to 14 cc. (Tsuchiya's reagent is 1.5 grams phosphotungstic acid dissolved in acid alcohol made by mixing 5 cc. of concentrated hydrochloric acid with 95.6 cc. of 95 per cent ethyl alcohol.)
3. Close the tube with the thumb and mix well by inverting slowly 3 times.
4. Centrifuge for 5 minues at 3500 r.p.m., or longer at less speed. (The precipitate must be so well packed that it can be drained without loss.)

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* Read before the Seventeenth Annual Convention of the American Society of Clinical Pathologists, June 9–11, San Francisco, Calif.

† If oxalated plasma is used add an equal volume of 0.4 per cent calcium chloride, mix and leave at 37°C. for at least 20 minutes. The fibrin can then be removed by rotating a spiked glass rod in the clot until the serum is expressed and the fibrin is tightly wound round the rod. Take 1 cc. of the diluted plasma serum for the determination. If it is desired, the removed fibrin can be measured by any of the standard procedures.

5. Pour off fluid and drain well by inverting tube on filter paper.‡

6. Wipe out excess liquid from inside of tube with filter paper, using care not to touch the precipitate.

7. Stand inverted tube on a watch-glass and place in an oven at 110°C. for 5 minutes. (The precipitate will drop out onto the watch-glass, or can easily be tapped out).§

8. Immediately place the watch-glass containing the precipitate cone on black glazed paper and, with a flexible, 3 inch, steel spatula smear the precipitate in a thin layer over the surface of the 2½ inch diameter watch-glass. Any particles that fall off onto the paper are returned to the watch-glass with a camel's hair brush.

9. Allow this smeared precipitate to stand in the air for about 3 minutes. (When running duplicates, the first specimen will be ready for the next step by the time the second specimen is smeared.)

10. Now scrape the dried precipitate *free from the surface* of the watch-glass (though not removing it from the watch-glass) and powder well by mashing with the spatula. This step is also done over black paper and drafts should be avoided.

11. Place the watch-glass again in the oven for 15 minutes.

12. Remove to a dessicator for 10 minutes.

13. Weigh on an analytical balance after transferring the powder to a tared watch-glass, again using care to work over black paper and to brush all particles from the paper to the watch-glass. It will be found that the powder does not gain weight while it is being weighed even though it is exposed to room air.

Under these conditions the observed weight of the precipitate is 170.4 per cent of the dry weight of protein it contains.

The calculation of total serum protein in terms of grams per 100 cc. of serum is given by the formula

$$\frac{\text{Observed weight in milligrams} \times 20}{170.4} \text{ equals grams per 100 cc.}$$

Thus if 60.6 mgm. is the weight of the precipitate from 0.5 cc. of serum the total serum protein is $\frac{60.6 \times 20}{170.4} = 7.11$ grams per 100 cc. of serum.

METHOD FOR SERUM ALBUMIN

1. Add 3 cc. of saturated ammonium sulphate to 3 cc. of serum or oxalated plasma, and, after mixing well, centrifuge for 15 minutes at 3500 r.p.m. If

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‡ In milky lipemic serums an extra step is taken at this point. Add 95 per cent alcohol to the precipitate, mix well with a glass rod and centrifuge for 5 minutes. Drain and proceed as indicated.

§ If the precipitate does not fall out of the tube while it is in the oven, take hold of the tube near its open end holding it horizontally and tap the tip of the tube gently on the table top. The precipitate will thus be detached and will slide down from the tip of the tube.

such a high speed is not available the separation can be more quickly effected if the material is distributed in four centrifuge tubes.

2. Transfer 2 cc. of the supernatant fluid (equivalent to 1 cc. of serum or plasma) into another 15 cc. centrifuge tube, add 7 cc. of 0.1 N sulphuric acid and 1 cc. of 10 per cent sodium tungstate. Mix and centrifuge for 5 minutes at 3500 r.p.m.

3. Decant the supernatant fluid and drain the tube by inverting it over filter paper.

4. Wash the precipitate of albumin by filling the tube with water and stirring it with a glass rod, centrifuge and drain.

5. Wash with water, centrifuge and drain.

6. Fill the tube with 95 per cent alcohol, mix well with a glass rod, centrifuge, drain and dry the lower part of the tube with filter paper.

7. Place the inverted tube over a watch-glass for 5 minutes in the oven at 110°C.

8. Remove the cone of precipitated albumin to a clean watch-glass, smear it over the surface and proceed as in the determination of total protein.

The calculation of serum albumin in grams per 100 cc. of serum is

$$\frac{\text{Observed weight in milligrams} \times 10}{170.4} \text{ equals grams albumin per 100 cc.}$$

THE DEGREE OF PRECISION OF THE METHOD

The reliability of the method was tested in three ways.

1. Serums were selected that contained the widest possible differences in protein content (3.48 grams per 100 cc. to 13.97 grams per 100 cc.) and repeated determinations were made in order to find whether the reliability varied with the amount of protein in the serum. Table 1 shows that the percentage error is independent of the quantity of protein taken for determination, except in the case of unusually low serum protein values where the error is slightly greater.

2. The difference between duplicate determinations on the same serum with this method was compared with the differences found in duplicate determinations by Barnett, Jones and Cohn with their gravimetric method.[1] In twelve duplicate determinations they found an average difference of 1.12 mgm. which is 1.9 per cent of the average amount of protein weighed. In thirty duplicate determinations with this method the average difference was 0.91 mgm. which is 1.7 per cent of the average amount of protein weighed.

3. In our hands the gravimetric method of Barnett, Jones and

Cohn gives more reliable results than those obtained with any other of the standard methods, but it is possible to increase its precision by washing the precipitate on the asbestos filter five times with hot buffer, ten times with hot 95 per cent alcohol and five times with absolute alcohol. With this additional washing the average difference between duplicates was reduced from 1.9 per cent to 0.9 per cent of the average amount of protein weighed. Duplicate determinations by this modified gravimetric method on the sera of thirty patients were carried out and compared with the weight of protein found in duplicate determinations on the same material by the new method. On the average the observed weights with the new method were 170.42 per cent of the modified gravimetric weights. The standard deviation of this

TABLE 1

NUMBER OF DETERMINATIONS	AVERAGE WEIGHT OF PROTEIN IN 0.5 CC. SERUM	AVERAGE DEVIATION FROM AVERAGE WEIGHT	AVERAGE DEVIATION AS PERCENTAGE OF AVERAGE WEIGHT
	mgm.	*mgm.*	*per cent*
20	119.0	2.1	1.7
8	63.4	0.8	1.2
8	52.3	0.7	1.3
8	40.8	0.7	1.7
8	29.6	0.7	2.3

average factor was ±3.37 per cent and the probable error in a single determination ±2.27 per cent. This is the basis of the factor 170.4 used in the calculation of total protein in grams per 100 cc. serum.

In another series of fifteen patients the albumin content of the serum was determined by the modified Barnett method and by the new method. On the average the new method weights were 171.42 per cent of the gravimetric with a probable error for a single determination of ±7.36 per cent. Since there is no statistically significant difference between 170.42 and 171.42, the factor of 170.4 has been used for the albumin as well as for the total serum protein.

Using this factor of 170.4 the results with the new method were expressed as grams per 100 cc. of serum and the error determined

on the assumption that the correct results were given by the modified gravimetric method. The data for total protein and albumin are given in table 2.

TABLE 2

NUMBER	TOTAL SERUM PROTEIN		TOTAL SERUM ALBUMIN	
	Modified gravimetric	New method	Modified gravimetric	New method
	grams per 100 cc.	*grams per 100 cc.*	*grams per 100 cc.*	*grams per 100 cc.*
1	7.58	7.50	5.58	5.70
2	7.52	7.42	5.21	5.35
3	7.31	7.32	5.16	5.28
4	7.31	7.18	5.00	5.13
5	7.30	7.23	4.63	4.65
6	7.26	7.14	4.28	4.46
7	7.26	7.55	4.02	4.10
8	7.16	6.98	3.92	4.28
9	7.08	7.14	3.72	3.89
10	7.04	7.06	3.31	3.50
11	6.89	6.90	3.26	3.48
12	6.79	6.90	3.08	2.69
13	6.66	6.38	2.91	2.90
14	6.63	6.72	1.90	1.66
15	6.46	6.68	1.70	1.55
16	6.44	6.45		
17	6.35	6.36		
18	6.18	6.43		
19	6.06	6.16		
20	6.01	5.88		
21	5.80	5.78		
22	5.42	5.24		
23	5.18	5.14		
24	5.11	5.13		
25	5.08	5.24		
26	4.69	4.64		
27	4.53	4.59		
28	4.43	4.38		
29	4.26	4.34		
30	3.86	3.75		

For the total protein the standard deviation is ± 0.13 per cent and the probable error ± 0.09 per cent. For albumin the standard deviation is ± 0.16 per cent and the probable error ± 0.11 per cent. Thus in subsequent analyses we may anticipate that

half the results will be within ±0.09 gram per 100 cc. of the results obtained by the modified gravimetric method for total serum protein and within ±0.11 gram per 100 cc. for albumin.

It is considered therefore that the new method can be recommended as yielding dependable results with very much less time and labor than are required for other gravimetric or Kjeldahl methods. A final test of the availability of the method for general use was made by giving two workers, who knew nothing of the method, the instructions and asking them to make repeated determinations on a serum of known protein content. One made four determinations and the greatest error was 2.9 per cent (i.e. with 7.50 grams per 100 cc. serum as the correct result his largest error was to estimate 7.61 grams per 100 cc.), the other made twelve determinations and his greatest error was 2.6 per cent. These are results that in our experience cannot be obtained by inexperienced workers with any of the standard methods.

CONCLUSION

1. A new method for the determination of total serum protein and of serum albumin is described that is based on the fact that protein precipitates from an acid alcohol solution of phosphotungstic acid can be very rapidly dried to a constant weight that bears a uniform relation to the weight of protein present in the precipitate.

2. Data are given on the reliability of the method and on its immediate availability for use.

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DEPROTEINIZING REAGENT; FOR BLOOD CHEMISTRY*

BENJAMIN KRAUTMAN

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The present widely used method for precipitation of protein and the preparation of filtrate for routine blood chemical analysis is that described by Folin-Wu. The tungstic acid filtrate as used by Folin-Wu[1] has thus far been the only one for the determination of all water soluble substances of blood. The special methods of precipitation advocated by some investigators (Somogyi;[2] Folin;[3] Herbert and Bourne;[4] Benedict;[5] Shaffer and Hartman)[6] seem to be neither necessary nor advantageous, except in such cases where a special study of blood is called for. In a large laboratory where hundreds of determinations are made daily any modification for the precipitation of blood proteins which would allow fewer steps in procedure and save laboratory space and do away with certain measurement errors would be a distinct advantage.

It is a common observation that if a number of bottles with sodium tungstate and dilute H_2SO_4, in the proportion as used in the Folin-Wu method, are allowed to stand whether at room temperature, 37 degrees Centrigrade, or on ice, a turbidity and a white precipitate invariably develops within a few days. The longer such a solution is allowed to stand, the less serviceable it is.

After observation of numerous combinations of the two compounds, a mixture was perfected which would not precipitate, become turbid, or change color, for an indefinite period of time. Buffering with sodium pyrophosphate and glacial phosphoric acid was necessary. Stock solutions of this reagent have been kept at room temperature, for the last two years and are still unchanged and serviceable. From this stock reagent, the working solution is obtained by dilution.

* Received for publication, May 15th, 1938.

METHOD FOR PREPARATION OF STOCK REAGENT SUFFICIENT FOR ONE LITER OF WORKING SOLUTION

1. Dissolve 5.6 gms. HPO_3 to 1 liter. Place 5 cc. of this sol. in 250 cc. flask.
2. Add 2.38 grams of sodium pyrophosphate;
3. Add to this mixture exactly 2.86 cc. of concentrated sulphuric acid, and while shaking add slowly 50 cc. of ten per cent of sodium tungstate (Baker's);
4. Place the flask in boiling water bath and shake *until solution is complete.*

TABLE 1

A DUPLICATE SET OF BLOOD SUGARS DETERMINED WITH THE FOLIN-WU METHOD[7]

	FOLIN-WU FILTRATE PER 100 CC. OF BLOOD	PREPARED DEPROTEINIZING FILTRATE PER 100 CC. OF BLOOD
	mgm.	*mgm.*
1	228	220
2	200	205
3	177	179
4	190	201
5	320	305
6	400	394
7	533	541

TABLE 2

A DUPLICATE SET OF BLOOD NON-PROTEIN NITROGEN DETERMINED WITH THE FOLIN-WU[8] METHOD

	FOLIN-WU FILTRATE PER 100 CC. OF BLOOD	PREPARED DEPROTEINIZING FILTRATE PER 100 CC. OF BLOOD
	mgm.	*mgm.*
1	37	35
2	29	31
3	51	58
4	48	45
5	98	90
6	183	191

5. Add 61.1 cc. of 10 per cent sodium tungstate; mix.
6. Add about *10 cc.* of distilled water, and store in glass stoppered amber bottle at room temperature.

To prepare working solution: Place the total volume of the above stock reagent in a one liter volumetric flask, rinse stock bottle with distilled water into volumetric flask and add distilled water q.s. 1000 cc. mark.

Each cubic centimeter of blood is added to 9 cc. of the working solution of prepared deproteinizing reagent, the mixture shaken until it turns brownish. The filtrate obtained is used in the same way and for the same determinations as the Folin-Wu protein free filtrate.

TABLE 3

A Duplicate Set of Blood Creatinines Determined with the Folin-Wu Method[7]

	FOLIN-WU FILTRATE PER 100 CC. OF BLOOD	PREPARED DEPROTEINIZING FILTRATE PER 100 CC. OF BLOOD
	mgm.	*mgm.*
1	1.30	1.28
2	2.50	2.35
3	4.10	4.51
4	2.93	3.15
5	3.14	3.50
6	10.5	11.50
7	12.6	11.90

TABLE 4

A Duplicate Set of Blood Uric Acids Determined with the Brown Method[8]

	FOLIN-WU FILTRATE PER 100 CC. OF BLOOD	PREPARED DEPROTEINIZING FILTRATE PER 100 CC. OF BLOOD
	mgm.	*mgm.*
1	2.94	3.10
2	3.06	3.40
3	3.10	3.30
4	5.30	5.40
5	2.50	2.60
6	6.61	6.70

TABLE 5

A Duplicate Set of Blood Sodium Chloride Determined with the Volhard-Whitehorn[7] Method

	FOLIN-WU FILTRATE PER 100 CC. OF BLOOD	PREPARED DEPROTEINIZING FILTRATE PER 100 CC. OF BLOOD
	mgm.	*mgm.*
1	316.0	357.0
2	423.3	418.2
3	602.1	595.0
4	484.2	498.5
5	459.3	473.4
6	397.8	408.0
7	571.4	558.0

The "tables" show comparative values on the same bloods, using the tungstic acid filtrate of Folin-Wu and a filtrate ob-obtained with the Deproteinizing Reagent.

For complete routine blood chemistry determinations 5 cc. of blood has proven to be most convenient. Exactly 5 cc. of blood withdrawn from patient's vein with an accurately calibrated 5 cc. syringe can be emptied into 45 cc. of prepared reagent in a *rubber stoppered* bottle and shaken for a few seconds. The laboratory receives the specimen ready for filtration; the filtrate is then ready for the determination of all water soluble substances of blood; such as N.P.N., Urea Nitrogen, Sugar, Amino Acid, Acetone Bodies, etc. This does away with preparation of citrated or oxalated bottles and minimizes to a certain extent interference in chemical determinations by these substances. It eliminates the necessity of pipetting blood from the specimen bottle, thus reducing error and saving time. It makes for less waste of blood; this is a factor to be considered in debilitated and anemic patients.

SUMMARY

1. A new reagent for preparation of protein free filtrate is discussed.

2. Its advantages are enumerated.

3. It is available for all determinations which can be done with Folin-Wu filtrate.

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HORTEGA'S SILVER IMPREGNATION METHODS*

Technique and Applications

WM. M. GERMAN

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In a preceding number of this journal I have described the technique and discussed the application of three of Hortega's silver impregnation tissue staining methods. I am herewith describing a group of three procedures by the same author, used for the demonstration of different types of connective tissue fibrils which are not revealed by the ordinary staining methods.

HORTEGA'S METHOD OF DOUBLE IMPREGNATION FOR CONNECTIVE TISSUE

A. Materials as before suggested.

B. Method.

1. Tissue fixed in formol.
2. Sections by freezing microtome (very thin).
3. Wash sections at least three times in distilled water.
4. Put sections in 10 cc. 2 per cent silver nitrate at room temperature.
5. Heat without cover over micro-burner very slowly at 40 to 45 degrees centigrade until light yellowish color develops.
6. Wash sections in distilled water.
7. Place sections in 10 cc. silver carbonate solution plus three drops of pyridine, at room temperature.
8. Cover with watch crystal and heat over micro-burner very slowly at 50 to 55 degrees centigrade, with occasional rotation of dish until a rich brown color develops.
9. Wash sections in distilled water.
10. Put sections in 10 per cent formol solution to reduce silver (about 1 minute).
11. Wash in distilled water.
12. Divide sections in two groups, (a) and (b).

(a) 1. Place sections in 5 per cent sodium hyposulphite solution 1 minute.
2. Wash sections in distilled water.
3. Mount sections on slide, drain excess water, blot.
4. Dehydrate by flooding slide with alcohol, drain, blot.
5. Clear by flooding slide with creosote, drain, blot.

* Received for publication July 5, 1938.

6. Mount in xylol free balsam with no. 1 cover glass.

(b) 1. Put sections in 10 cc. 0.2 per cent gold chloride solution at room temp.
2. Heat over micro-burner very slowly, without cover, until the sections are dark.
3. Wash sections in distilled water.
4. Place sections in 5 per cent sodium hyposulphite solution 1 to 2 minutes with slight rotation of sections.

FIG. 1. PHOTOMICROGRAPH SHOWING USE OF HORTEGA'S DOUBLE IMPREGNATION METHOD

Visualizes complicated system of connective tissue fibrils as a protective mechanism in the wall about a gastric ulcer.

5. Wash in distilled water.
6. Mount section on slide, drain excess water, blot.
7. Dehydrate by flooding slide with alcohol, drain, blot.
8. Clear by flooding with creosote, drain, blot.
9. Mount in balsam with no. 1 cover glass.

Results: This method is useful for demonstration of some of the finer connective tissue fibrils in connective tissue, inflammatory

tissue, sarcomata of fibroblastic origin and the fibrillar structure in nerve sheaths. In series A the fibrils stain deep brown to black while muscle cells and epithelial cells stain a golden brown. In series B the fibrils stain black while muscle and epithelial cells stain a mahogany red (fig. 1).

FIG. 2. PHOTOMICROGRAPH SECTION LIVER SHOWING SELECTIVE VISUALIZATION OF SYSTEM OF INTERCELLULAR FIBRILS WHICH SUPPORT LIVER CELLS AND CORDS

HORTEGA'S METHOD OF DOUBLE IMPREGNATION WITH POTASSIUM PERMANGANATE FOR STAINING CONNECTIVE TISSUE

A. Materials as before suggested.

B. Method:

1. Tissue fixed in formol.
2. Sections cut by freezing microtome (very thin).
3. Wash sections twice in distilled water.
4. Place sections in 10 cc. of 1:400 potassium permanganate at room temperature for 10 minutes.
5. Wash sections twice in distilled water.
6. Place sections in 5 per cent oxalic acid until completely decolorized.
7. Wash sections twice in distilled water.

8. Place sections in 10 cc. 2 per cent silver nitrate at room temperature.
9. Heat without cover over micro-burner, very slowly, 50–55 degrees centigrade until sections assume a pale yellowish color.
10. Wash sections in distilled water.
11. Place sections in 10 cc. silver carbonate solution at room temperature.
12. Cover dish with watch crystal and heat very slowly over micro-burner at 50–55 degrees centigrade until deep rich brown.

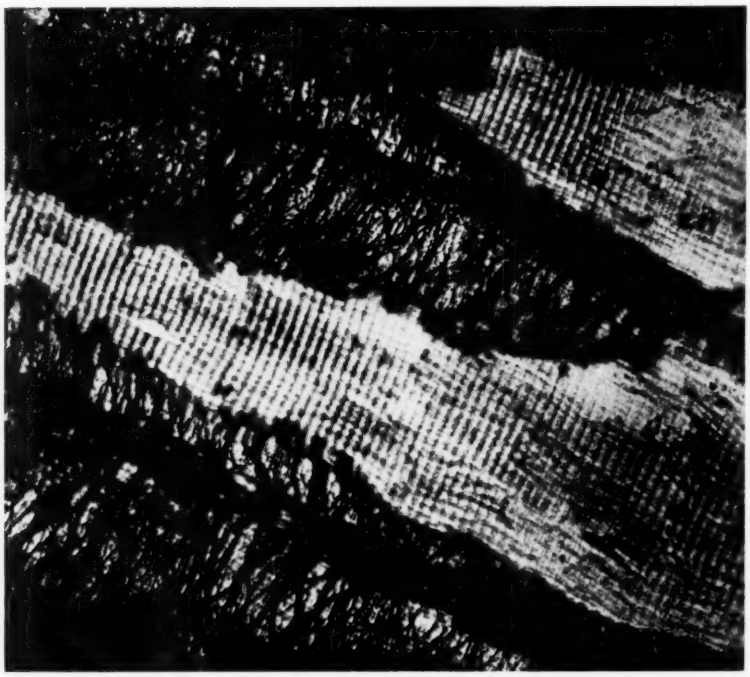

FIG. 3. PHOTOMICROGRAPH, OIL IMMERSION, STRIATED MUSCLE, SHOWING SELECTIVE VISUALIZATION OF INTRICATE SYSTEM OF ENDOMYSIUM WHICH COMPLETELY SURROUNDS EVERY MUSCLE CELL WITH A LACE WORK OF FIBRILS

13. Wash in distilled water.
14. Place sections in 10 per cent formol solution for 1 minute.
15. Wash sections in distilled water.
16. Divide sections into two groups, (a) and (b).
    (a) 1. Place sections in 5 per cent sodium hyposulphite solution 1 minute.
        2. Wash in distilled water.

3. Mount section of slide, drain excess water, blot.
4. Dehydrate by flooding slide with alcohol, drain, blot.
5. Clear by flooding with creosote, drain, blot.
6. Mount with balsam and no. 1 cover glass.

(b) 1. Place sections in 10 cc. 1:500 gold chloride solution at room temperature.
2. Heat without cover very slowly over micro-burner until sections are dark.
3. Wash sections in distilled water.
4. Place sections in 5 per cent sodium hyposulphite solution one minute.
5. Wash in distilled water.
6. Mount section on slide, drain excess water, blot.
7. Dehydrate by flooding slide with alcohol, drain, blot.
8. Clear by flooding slide with creosote, drain, blot.
9. Mount in balsam with no. 1 cover glass.

Results: This method demonstrates all the connective tissue elements. It is exceptionally useful in demonstrating the finer fibrils which are not seen by the ordinary methods, such as inter-epithelial fibrils, the fibrillar network supporting various gland cells such as is seen in the liver, and the endomysium fibrils which form a complete network about each and every muscle cell. It demonstrates nerve sheath structures and neurofibrils (figs. 2 and 3).

METHODS OF GALLEGO

The following two methods do not, strictly speaking, belong in the general group of Hortega's impregnation procedures. They are used extensively, however, in the Cancer Institute of Madrid, and have their definite purposes. Using frozen sections of formol fixed tissue, they, of course, do not lend themselves to mass production as does the well known H and E technique.

1. Gallego's general tissue stain

A. Materials, as before suggested.
B. Technique:
1. Tissue fixed in formol.
2. Freshly cut sections by freezing microtome (thin).
3. Wash sections in distilled water.

4. Stain sections in acetic-fuchsin-solution for 2 minutes at room temperature. Acetic-fuchsin solution: distilled water, 10 cc.; glacial acetic acid, 1 drop; Ziehl's fuchsin, 10 drops.
5. Wash in distilled water.
6. Differentiate by placing sections in acetic-formol solution for 5 minutes at room temperature. Acetic-formol solution: distilled water, 10 cc.; conc. formaldehyde, 2 drops; glacial acetic acid, 1 drop.

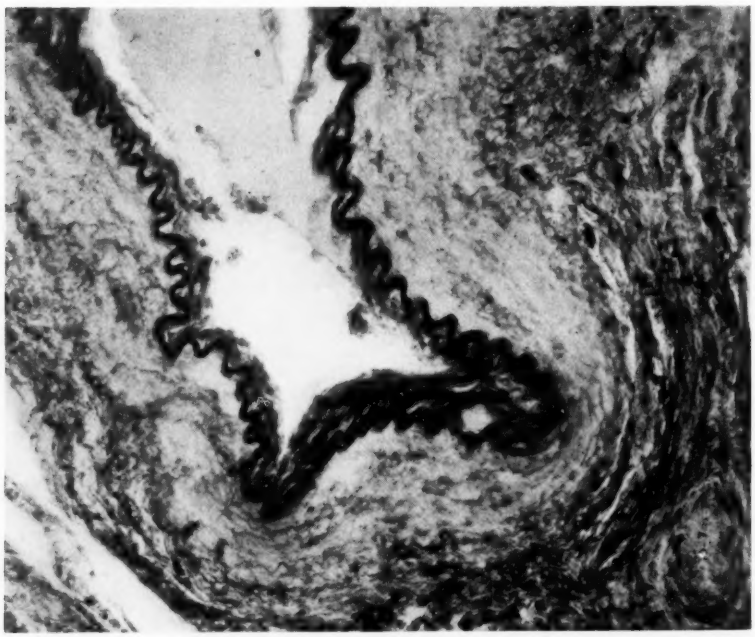

FIG. 4. PHOTOMICROGRAPH SECTION OF VESSEL WALL SHOWING DIFFERENTIAL VISUALIZATION OF ELASTIC TISSUE, USING GREEN FILTER

In the section a brilliant red elastic membrane is seen against a green background of collagenous tissue.

7. Wash sections in distilled water.
8. Mount section on slide, drain excess water, blot.
9. Counterstain section by flooding slide with picro-indigo, drain, blot.
10. Dehydrate quickly by flooding slide with absolute alcohol, drain and blot.
11. Clear by flooding slide with carbol-xylol, then xylol, drain and blot.
12. Mount with Canada balsam and cover glass.

Results: This is a brilliant general tissue stain. All nuclei are stained a magenta red, epithelial cytoplasm a yellowish pink, connective tissue is stained a brilliant green, muscle is olive green, cornified epithelium and blood a grass green. The general differentiation of the various tissue structures is striking.

2. Gallego's method for differential staining of elastic tissue

A. Materials as before suggested.
B. Technique:
1. Tissue fixed in formol.
2. Freshly cut sections by freezing microtome (very thin).
3. Wash sections in distilled water.
4. Sensitize sections in formol-nitric-ferric solution for 30 seconds. Formol-nitric-ferric solution: water, 10 cc.; conc. formaldehyde, 2 drops; 50 per cent iron sesquichloride, 1 drop; conc. nitric acid, 1 drop.
5. Without washing, transfer sections to acetic-fuchsin solution for 5 minutes. Acetic-fuchsin solution: water, 10 cc.; glacial acetic acid, 1 drop; Ziehl's fuchsin, 15 drops.
6. Wash sections in distilled water.
7. Differentiate sections by placing in above formol-nitric-ferric solution for 1 to 5 minutes (depending on the sections).
8. Wash in distilled water.
9. Mount section on slide, drain excess water, blot.
10. Flood slide with counterstain picro-indigo for 1 min., drain, blot.
11. Dehydrate by flooding slide with absolute alcohol, drain, blot.
12. Clear by flooding slide with carbol-xylol, then xylol, drain, blot.
13. Mount with Canada balsam and cover-glass.

Results: This method effects a complete differentiation between elastic and collagenous connective tissue. The elastic tissue is stained a brilliant fuchsin red while collagenous connective tissue is stained a brilliant green. Such results lend themselves to striking photographic demonstrations, even in black and white, making use of light filters. Nuclei are stained lavender, cell cytoplasm a yellowish pink, cornified epithelium and sebaceous glands are stained yellow (fig. 4).

THE ISOLATION AND IN VITRO TESTING OF PATHOGENIC TYPES OF NON-EXOTOXIC STREPTOCOCCI*

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Until quite recently there were no satisfactory methods for differentiation of non-exotoxic streptococci on the basis of probable pathogenicity. "Hemolytic" types were considered pathogenic because they were associated with acute diseases, and non-hemolytic types were considered non-pathogenic because they were associated with chronic, less dramatic diseases. Some workers considered "viridans" strains as being the incitants of chronic disease. These relationships are only generally true. Hemolytic streptococci may be non-pathogenic. Gamma types may produce disease, and "viridans" cultures may be lacking in pathogenicity. For these reasons, Brown's classification, which employs blood agar plates and is useful for tentative separation, should not be used as an indicator of pathogenicity, particularly when hemolysis is determined by plating the culture on the surface of a blood agar plate, as is often done, and incubating in air.

Taxonomic relationships have been clarified by the investigations of Sherman and his associates, which have been brought up to date by Sherman.[11, 12] Chapman, Berens and Nilson[2] differentiated cultures on the basis of resistance to injurious agents, which they showed to be parallel with pathogenicity of the live and killed cultures for rabbits. Although tests were not made on exotoxic streptococci, a similar relationship holds for that group also, as had been shown by Todd,[13] Hare[7] and Downie.[6] The principle is not applicable to enterococci.

The isolation and testing of resistant non-exotoxic types is not a simple procedure. Search for better isolation methods resulted in certain refinements, and it is hoped that publication of this report will help others who have had difficulty in securing resistant cultures.

* Received for publication, June 28th, 1938. Aided by grants from the Ophthalmological Foundation, Inc.

It was stressed that the cultures to be tested for resistance should be relatively free from dissociants. These must be separated as rapidly as possible because many of the cultures lose their resistance within a few days (table 1). It is unwise to attempt absolute purification of all cultures because some strains dissociate continuously, and delay in testing them may result

TABLE 1

DEGENERATION OF RESISTANCE OF STREPTOCOCCI

NUMBER OF DAYS SINCE THE CULTURE WAS OBTAINED FROM THE BODY		NUMBER OF CULTURES
4 days	7 days	
8+	8+	4
	7+	5
	5+	4
7+	8+	1
	7+	2
	6+	4
	5+	3
	4+	5
6+	7+	1
	6+	1
	5+	2
	4+	5
5+	4+	1
4+	4+	1
	0	1
3+	2+	1
	0	3

in further degeneration. Pure cultures may be secured from single colonies in most cases.

The resistance tests were made as follows. A loopful of the culture was added to a tube of each of the following: (1) 0.5 cc. of 0.3 per cent sodium bicarbonate; (2) 0.5 cc. of 0.5 per cent sodium bicarbonate; (3) 0.5 cc. of 1:200,000 hexylresorcinol; and (4) 0.5 cc. of 1:125,000 hexylresorcinol. After thorough shaking, a loopful from tube (1) was streaked about 2 cm. on blood agar to serve as a control. The tubes were put in the incubator at 37°C. At the end of 1 hour a loopful from tubes (1) and (3) was streaked on the same blood agar plate as the control. At the end of 2 hours, a loopful from tubes (2) and (4) was streaked on the plate also. The blood agar plate was incubated overnight

and the growths compared with the control. If the growth was undiminished after treatment with the bacteriostatic agent, it was reported as ++++. If it was slightly reduced, it was recorded as +++. If it was moderately reduced, it was recorded as ++. If it was markedly reduced, or *if there was no growth, not even in the control streak*, it was recorded as negative (0). The results from tubes (1) and (2) were added, giving a maximum of 8+. The results from tubes (3) and (4) were added also. There should be no serious discrepancy between the totals of (1) and (2), and (3) and (4).

Streptococci that react positively to the in vitro tests (8+ and 7+) will be referred to as resistant streptococci.

Resistance to the bactericidal action of fresh, diluted, defibrinated guinea pig blood and to the chemical agents just described

TABLE 2

(Adapted from Rawls and Chapman, 1935)

RELATION BETWEEN RESISTANCE TO FRESH BLOOD, AGGLUTINABILITY, AND THE ARTHRITIS-PRODUCING POWER OF STREPTOCOCCI

NUMBER OF CULTURES	RESISTANCE TO FRESH BLOOD	AGGLUTINABILITY	REQUIRED TO PRODUCE ARTHRITIS IN RABBITS			
			Mean interval	Mean maximum dose	Mean total volume	Mean number of injections
			days	*cc.*	*cc.*	
19	+	0	25	4.1	28.2	10
7	+	+	34	4.7	34.	12
10	0	+	54	9.6	61	13
9*	0	0	30	15.0	100	14

* Only 3 rabbits in this group developed arthritis.

was parallel with lethal effects of live and killed cultures.[2] Rawls and Chapman[3] showed that streptococci which resisted the bactericidal action of fresh, diluted, defibrinated guinea pig blood produced arthritis in rabbits more frequently, more quickly, and with less culture than strains which were susceptible to the bactericidal action (table 2).

Additional confirmation of the possible value of resistance tests was shown by Chapman and Lieb[4] who found that the average number of B. coli in feces was inversely proportional to the presence of resistant streptococci in the specimens. This relationship was confirmed in another series (C.B.) (table 3).

It should be re-emphasized that the relationship between

pathogenicity and resistance to injurious agents does not hold for enterococci. Most of the cultures of enterococci were non-pathogenic for rabbits although they were highly resistant to chemical and physical agents. Most enterococci are considered non-pathogenic for human beings also, but infections have been attributed to this organism. Because of the anomalous behavior so far as the resistance tests are concerned, this group must be eliminated from the tests.

In several papers Sherman has criticized the use of the term "enterococcus" in the loose form in which it is often used, i.e. to connote an intestinal streptococcus. There has existed an impression that we, too, had confused the taxonomic relationships of the group. Because we were interested primarily in

TABLE 3

RELATIONSHIP BETWEEN THE PRESENCE OF RESISTANT STREPTOCOCCI IN FECES AND THE AVERAGE NUMBER OF B. COLI

STREPTOCOCCI ISOLATED	NUMBER OF SPECIMENS	AVERAGE NUMBER OF B. COLI
		millions per 100 gms. dry feces
Resistant (7+ and 8+)	64	57,159
Borderline (6+) or sensitive (5+ and less)	32	162,500
No streptococci isolated	37	667,659

pathogenicity, we did not classify streptococci on the basis of "species" but referred to all members of the anomalous group as "enterococci" and suggested growth on Levine's eosine methylene blue medium as a differential test. Because Streptococcus lactis, like the enterococcus, is resistant to chemical agents but is not pathogenic for rabbits, we considered the two organisms "closely related," so far as the resistance tests were concerned. Sherman has described differential characteristics.

Rosenow[9] showed that seasonal variations influenced the cataphoretic velocity of streptococci. Pathogenic types were more easily isolated in our laboratory during inclement weather. To facilitate isolation of pathogenic streptococci, particularly during good weather, improved isolation methods were sought. Theoretically, it should be possible to isolate pathogenic types by add-

ing injurious agents to the culture medium. Several thousand freshly isolated cultures were tested, using different concentrations of more than 100 dyes, also metal salts and other compounds. However, the results did not appear to be satisfactory. There was an accuracy of about 65 per cent when pure cultures were plated directly on 0.10 per cent sodium bicarbonate blood agar, 1:15,000 hexylresorcinol blood agar, 0.026 to 0.032 per cent neutral red in starch agar and 0.0004 per cent methylene violet (safranin type) in starch agar.

There are several possible explanations for the failure to secure more accurate results. It was shown[8] that the resistance of a streptococcus to the bactericidal action of fresh, diluted, defibrinated guinea pig blood was not so specific in the presence of culture media. To eliminate this non-specific effect, cultures were subjected to the action of 0.3 per cent sodium bicarbonate for 1 hour and plated on bicarbonate blood agar. Quantitative tests indicated that the number of colonies was proportional to the resistance of the cultures. With a more extensive series of tests, however, the results did not agree with resistance tests in about 15 per cent of the cultures. This error was attributed to the injurious agent having a bacteriostatic, rather than a bactericidal action. After the initial bacteriostatic action in the bicarbonate solution, the organisms grew on blood agar as if they had not been injured. The specific effect noted when the cultures were streaked on blood agar in the resistance tests was attributed to the carrying over of the injurious agent.

The results were less specific when mixed cultures were tested, although they were better than with simple blood agar isolation (table 4). However, the method was abandoned because some resistant strains were not recovered.

A further difficulty was encountered. Streptococci degenerated rapidly after isolation, as claimed by Rosenow frequently. This was demonstrated on numerous occasions by means of the quantitative resistance tests (see table 1). Many strains, which gave maximum resistance immediately after isolation, were completely lacking in resistance when tested a few days later. The tendency to degenerate varied with the culture, and ap-

peared to be caused by the effect of oxygen because it could be reduced by maintaining the culture under mineral oil. Rosenow[10] claimed that anaerobic cultivation preserved the neurotropic type of cataphoretic velocity. This is in agreement with our findings.

Because of these difficulties, the isolation methods of Chapman and Rawls[5] were modified as follows. Bacto brain heart infusion was used because resistance of the cultures to sodium bicarbonate and hexylresorcinol was parallel with lethal effects on rabbits when the cultures were grown in this medium. Other liquid media gave less satisfactory results. Bacto tryptose agar was used as a base for blood agar because it gave much more luxuriant growth of streptococci than other base media. Dry swabs of

TABLE 4

ISOLATION OF RESISTANT STREPTOCOCCI FROM CRUDE CULTURES

METHOD USED	TOTAL "STRAINS" ISOLATED	PER CENT GIVING 6+ TO 8+ RESISTANCE
Direct plating from brain heart infusion culture..	134	34.3
Mix 1 loopful of culture with 0.5 cc. of 0.3 per cent $NaHCO_3$ for 1½ hours. Plate 1 loopful on blood agar and incubate overnight.....	157	60.5
Same, but 1½ hours exposure to $NaHCO_3$.....	49	75.5

material from various parts of the body, mailed from Southern states during the hottest part of the summer, were still satisfactory for the isolation of resistant strains. The swab of material suspected of containing pathogenic streptococci was allowed to soak a few minutes in Bacto brain heart infusion. It was then rubbed lightly on tryptose blood agar and returned to the brain heart infusion. The blood agar was spread by means of a glass spreader which was then rubbed over a second tryptose blood agar plate. The two plates were used for method 1. The brain heart infusion culture was used for method 2 which was run simultaneously.

Method 1: The tryptose blood agar plates were incubated anaerobically. The modified Varney jar[1] was used because it was simple and could be set up rapidly. The following day single

colonies were fished into separate tubes of brain heart infusion and incubated overnight. The growths were tested for purity, resistance, and power to grow on Levine's medium.

Method 2: After overnight incubation, the swab in the brain heart infusion was stirred to set free the growth adhering to it, and discarded. The culture was allowed to settle ½ hour and a loopful from the upper portion was plated on tryptose blood blood agar and incubated anaerobically. The following day, single colonies were fished into separate tubes of brain heart infusion. If no cultures showing 8+ or 7+ resistance were isolated by method 1, then cultures from method 2, which were finished 1 day later, were tested for purity, resistance, and growth on Levine's medium.

A series of 44 crude (mixed) cultures was tested by these two isolation methods and both gave similar results in 26 cultures. Method 1 was better in 10, and method 2 was better in 8, the discrepancies being attributed to the error of random sampling. Therefore, it is suggested that the combination of the two methods be used.

SUMMARY

We have attempted to describe difficulties which prevent selective isolation of probable pathogenic, non-exotoxic streptococci. The greatest difficulties were caused by the loss of specificity of inhibitive agents when used for mixed cultures, also the lability of the freshly isolated cultures.

An improved method has been described by which resistant (probable pathogenic) strains may be isolated from mixed cultures within 3 to 4 days.

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A CLINICAL BACTERIOLOGICAL TEST FOR THE RECOGNITION OF C. WELCHII IN WOUNDS*

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In order that the laboratory may be of service to the clinician in recognizing the presence of C. welchii in wounds in the early stages of gas gangrene or in advance of the development of definite clinical symptoms, it is necessary that the laboratory methods employed consume the least possible time and that they

* Received for publication June 21, 1938.

be as nearly specific as the limitations of biological methods will permit.

In a previous publication,[1] we have reported some advantages of the addition of fresh muscle tissue or sterile powdered iron to skim milk for demonstrating the production of a stormy fermentation of the milk by this organism. When either of these substances is added to the milk, the resulting stormy fermentation is more easily recognized and is produced in a shorter time and with a smaller inoculum than when milk alone is used. An objection may be raised to the use of fresh tissue because of the reported presence of C. welchii in normal tissue. If the cultures are properly controlled, the possibility of error from this cause is slight, but it can not be entirely discounted. When powdered iron is used this possibility is eliminated.

However, there is another difficulty which is encountered in mixed cultures—as are always found in gangrenous wounds, and which is not eliminated by either tissue or iron. As we have shown in the previous paper, some other organisms, notably streptococcus fecalis and streptococcus mitior, may interfere with the development of C. welchii unless the latter is definitely predominant. This interference may result in an atypical fermentation or in the complete suppression of the C. welchii. It would seem desirable, then, to find a medium in which the growth of these organisms would be inhibited or in which, at least, their effect on the growth and activity of the C. welchii would be minimized.

In 1924–25 Wilson and Blair[2, 3] reported the production of hydrogen sulfide from sodium sulfite by C. welchii in a glucose agar medium and the production of a black discoloration of the medium when ferric chloride was added. This characteristic they considered to be sufficiently specific for the recognition of C. welchii in water samples.

We have undertaken to determine (1) whether the production of H_2S can be demonstrated in a liquid milk medium as well as in a glucose agar medium, (2) whether this character might have advantages over the stormy fermentation of milk which we have previously used as a diagnostic character, and (3) whether this reaction is sufficiently specific to be valuable in diagnosis.

MEDIUM

As a basic medium, we have used skim milk, tubed in 10 cc. portions and sterilized in the autoclave at ten pounds pressure for fifteen minutes on two successive days. Just previous to inoculation, this milk was heated in a water bath, cooled, and to it were added 0.1 cc. of an 8 per cent solution of ferric chloride and a sterile 20 per cent solution of sodium sulfite in varying amounts. (The ferric chloride may be added after the cultures are incubated, if desired.)

Seven strains of C. welchii were used, three of which were type cultures and the remainder were isolated from infected wounds.

EFFECT OF SODIUM SULFITE ON C. WELCHII

When sodium sulfite was used in the proportion of 1 to 10 cc. of milk containing ferric chloride, C. welchii consistently gave good growth and black discoloration of the milk. Serial dilutions of C. welchii were made and inoculated into the medium in such manner that the series of tubes received diminishing numbers of organisms—an average of 100,000,000; 10,000,000; 1,000,000, etc. per tube. The results compared favorably with the stormy fermentation of iron-milk both in the number of organisms and the time required to produce the reaction. With some strains of organisms the reduction was evident in a shorter time or with fewer organisms than was stormy fermentation. With other strains the opposite was true. Reduction is generally evident within 12 hours if the inoculum contains 10,000,000 organisms, and frequently with as few as 100,000 organisms. In 24 hours, reduction is produced with 10,000 or often with smaller numbers of organisms, and 100 or less organisms will usually cause evident reduction within 36 hours.

Since the several strains of C. welchii used showed variations in their sensitivity to this concentration of sodium sulfite, observations were made of the effect of varying the amount of this solution added to the milk. Four strains of C. welchii were tested with concentrations of sodium sulfite ranging from 0.1 to 2.5 cc. of the 20 per cent solution to 10 cc. of milk. With 0.1 cc., the milk showed stormy fermentation and a dark grey rather than a typically black discoloration. More than 1.5 cc. gave noticeable inhibition of the C. welchii, in some cases manifested by the increase in time required for the reaction and in others by the complete suppression of growth. In all cultures examined by stained smears, those containing more than 1 cc. of sulfite showed a decided reduction in the number of organisms even though the medium was blackened. With 1 cc. or less sulfite there was no decided difference in the number of organisms in the cultures after incubation, irrespective of the number of organisms in the inoculum.

The optimum concentration, then, is 0.5 to 1 cc. and since the higher concentration would be expected to give more inhibition of other organisms, 1 cc. has been used throughout the remainder of the work.

EFFECT OF OTHER ORGANISMS ON THE REDUCTION OF SODIUM SULFITE BY C. WELCHII

While streptococcus fecalis does exert some inhibitive action on C. welchii in mixed cultures of these organisms in this medium, the action is definitely less pronounced than in tissue-milk or iron-milk. Stained smears of the sulfite-milk cultures show the growth of streptococci to be sparse. Attempts to eliminate or decrease the unfavorable effects of streptococci by raising the pH of the medium by means of NaOH or by phosphate buffers were not satisfactory.

Miscellaneous cultures inoculated with C. welchii and mixed organisms from wounds and feces showed, in sulfite-milk, almost pure cultures of C. welchii and typical black discoloration, while control cultures in iron-milk showed mixtures of organisms with or without stormy fermentation according to the proportion of C. welchii present in the inoculum.

SPECIFICITY OF THE REACTION

A number of anaerobic spore-formers (C. tetani, C. sporogenes, C. histolyticum, C. bifermentous, C. tertium, C. oedematiens, C. fallax, C. tetano morphum, C. sphenoides, C. butyricum iodophilum, C. botulinum, C. tyrosinogenes, C. multifermentous, C. saccharobutyricum, C. saprogenes canis, Vibrion septique, Bacillus of lamb dysentery, and a butyricum from sauerkraut spoilage) were cultured in this medium. Growth of most of these organisms was entirely suppressed. While others showed sparse growth, only two showed any evidence of discoloration of the medium. C. saprogenes canis sometimes showed a few black spots in the bottom of the tube at the end of 72 hours. However, this reaction was so delayed and so slight that the possibility of confusing this with a C. welchii reaction need cause little concern. C. butyricus iodophilum, with a heavy inoculation, will in some cases cause a blackening of sulfite-milk, although the reaction is not constant. The growth of C. butyricus iodophilum is sparse as seen by stained smears as contrasted with the heavy growth of C. welchii. C. butyricus iodophilum can be suppressed by the addition of 0.5 to 1 cc. of a pH 7.4 phos-

phate buffer to the culture. However, the medium is then less favorable for the growth of the C. welchii and its use is not advisable.

From bismuth sulfite plates used for the isolation of the typhoid bacillus a variety of colonies were picked and planted in sulfite-milk. These included mostly colonies which showed some degree of darkening of the bismuth sulfite medium. Twenty-six colonies were used, including B. typhosus, B. coli, staphylococci, streptococci, diphtheroids, and a variety of Gram-negative bacilli. The streptococci and staphylococci showed a few organisms by stain and the remainder showed no growth. In no case was there any evidence of reduction.

Miscellaneous facultative anaerobic spore-formers and mixed organisms from non-gangrenous infected wounds were cultured with no evidence of reduction.

SUMMARY AND CONCLUSIONS

The addition of 1 cc. of a 20 per cent solution of sodium sulfite and 0.1 cc. of an 8 per cent solution of ferric chloride to 10 cc. of skim milk forms a favorable medium for the growth of C. welchii. The reduction of the sodium sulfite with the consequent blackening of the medium gives a constant and easily recognizable characteristic either in pure or mixed cultures. This is the highest concentration of sodium sulfite which can be safely used without danger of inhibiting the growth and activity of the C. welchii.

Streptococci, which interfere with the stormy fermentation of tissue-milk or iron-milk, by C. welchii, were largely suppressed in sulfite-milk. There was some evidence of inhibition in the sulfite-milk but it was definitely less than in the other media.

Of the many other organisms studied, only one gave reduction of the sodium sulfite which might be confused with the reduction by C. welchii. C. butyricus iodophilum, when the inoculum contained a large number of organisms, gave a greyish or sometimes black discoloration of the milk. This reaction was not constant and growth of this organism was sparse as shown by stained smears. In such cases, the organisms can be distinguished by their morphology and motility.

We believe that sulfite-milk is a satisfactory medium and is sufficiently specific to be a valuable addition to the diagnostic media previously employed.

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A SPRING CLIP FOR THE BLOOD COUNTING CHAMBER*

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The spring clips for holding the cover slip in its correct position upon the counting chamber, described below, were designed and constructed in an effort to increase the accuracy of red and white blood cell counts which are not infrequently influenced by the manipulations necessary during the filling of the chamber or in transferring the chamber to the stage of the microscope.

One manufacturer provides clips which hold the cover slip in place on the counting chamber and in a measure obviate movement of the cover slip which may rise as the dilute blood flows beneath it to fill the chamber. However these clips do not fulfill all the requirements. (1) They are frail and soon get out of shape, thus exerting unequal pressure upon the cover slip. (2) They are somewhat difficult to attach without finger marking the cover slip. (3) They are apt to become displaced when the counting chamber is moved along by the mechanical stage.

* Received for publication July 16, 1938.

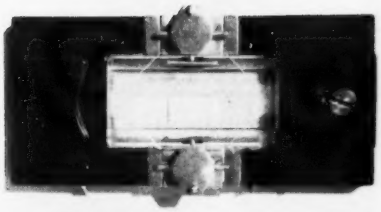

FIG. 1. Shows both clips in position and the counting chamber ready for use. It reveals the clips in true proportion to the counting chamber.

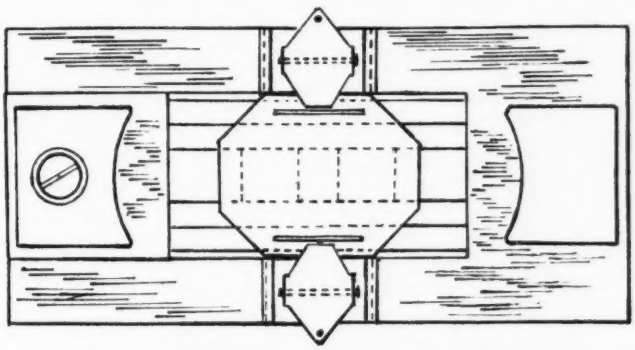

FIG. 2. An enlarged plan showing with more detail the clips in position

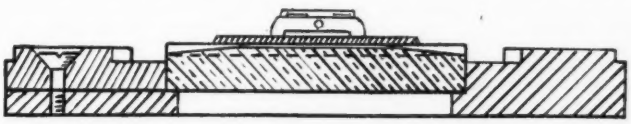

FIG. 3. An enlarged cross section of the chamber showing one of the clips in position and the "fingers" holding the cover slip in place on the shoulders. The "fingers" pivot on a centre pin which insures an equalization of pressure on each side of the moat.

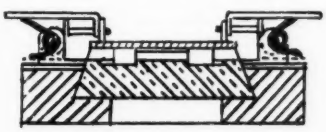

FIG. 4

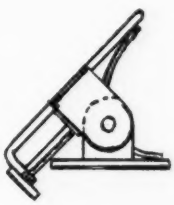

FIG. 5

FIG. 4. An end section showing both clips in position, and the springs, which exert just enough pressure to permit the "fingers" to hold the cover slip in place without distortion.

FIG. 5. An enlarged drawing of a clip as it looks when removed to facilitate the cleaning of the counting chamber upon the completion of a count.

In order to overcome these disadvantages and to provide clips positive in their action, the clips shown in figures 1, 2, 3, 4 and 5 were constructed. These clips have been adapted to the Levy-Hausser bakelite frame, counting chamber as shown and are also being adapted for use with any of the popular all glass type chambers.

These clips can be slipped on to the chamber in about two seconds and are retained in position by small runways which can either be permanent or detachable fittings to the counting chamber.

For the past six months our counting chambers have been equipped with the above described clips and have been in constant use in the laboratory. They have proved to be of great help, eliminating the possibility of moving the cover slip when filling the counting chamber and permitting consistent cell distribution.

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BLOOD TRANSFUSION EQUIPMENT AND SOME REACTION PREVENTION FACTORS*

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During the past fourteen months at the Graduate Hospital an attempt has been made to simplify transfusion equipment and to eliminate some reaction producing factors. The apparatus to be described, because of its greater efficiency and economy, has now entirely supplanted the original technique which appeared in a recent issue of the American Journal of Clinical Pathology.

Blood is drawn from a donor with the aid of mouth suction, as shown in figure 1. The equipment for blood taking is shown in detail in figure 2. (The needles are 15, 16 and 18 gauge; ampule of 1 per cent novacaine with syringe and needle; 20 cc. syringe, with oxalated tube for typing and cross-agglutination

* Received for publication November 25th, 1938.

and plain tube for serologic studies; flask with 70 cc. of 2.5 per cent sodium citrate; sterile paper cap for covering flask, which is supplemented by a cellophane cover when blood is stored; mouth suction unit with Murphy type bulb and cotton filter, tubing, and glass adapter.)

Blood is administered with the unit shown in figure 3. This consists of a specially designed filter of heavy Pyrex glass which permits air to enter flask as blood is filtered through three thick-

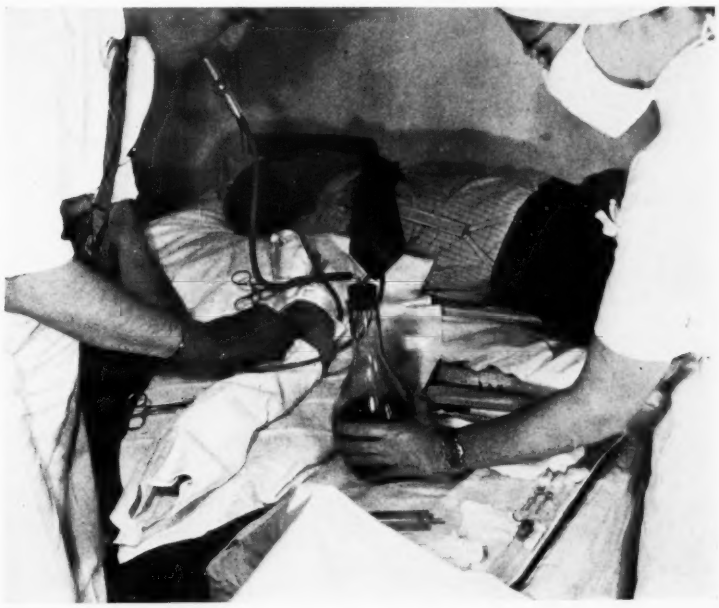


FIG. 1. COLLECTION OF BLOOD FROM DONOR

nesses of 32–28 bandage gauze; a one-holed rubber stopper; tubing; glass adapter and 18 gauge needle. Details of construction of the filter are shown in figure 4. In actual use, the filter is covered with gauze as in figure 5. The filter and stopper are inserted into the blood-containing flask, and the edges of the stopper are folded over the lip of the flask. Figure 6 shows the administration unit assembled. The rate of flow from the inverted flask is controlled by the stopcock. Air enters the flask

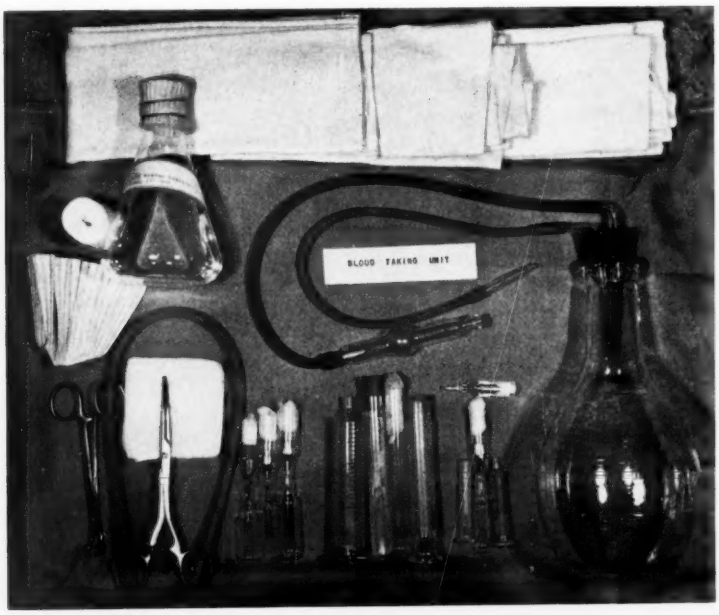


FIG. 2

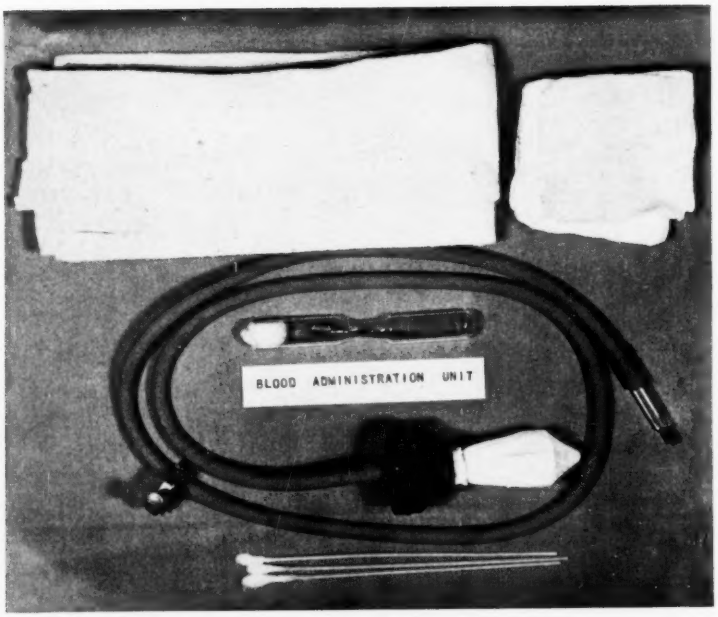


FIG. 3

FIG. 4. SPECIAL FILTER

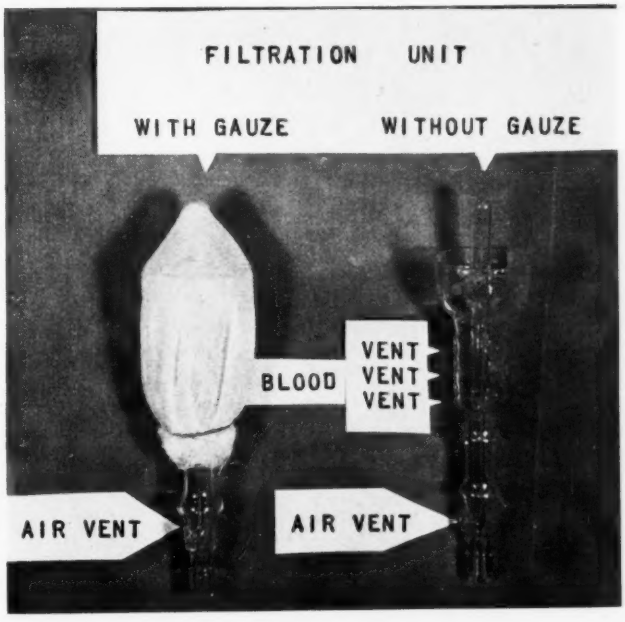


FIG. 5

through the air vent on the filter and prevents the production of a vacuum as the blood flows out.

All glass equipment is of heavy Pyrex and has withstood the strain of routine handling by many individuals in an active general hospital with minimal breakage.

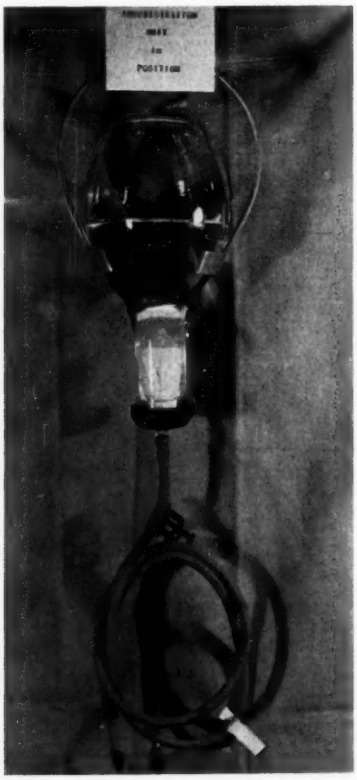


FIG. 6

The problem of prevention of reactions has been an interesting one and from the technical standpoint several factors seem to be important:

1. *Avoid heating the blood artificially.* Blood has been given immediately after removal from the refrigerator without reaction.

2. *Avoid handling the blood,* particularly if it has been refriger-

ated. Blood is a fragile fluid and cellular disintegration is decreased by not transferring to other containers and preventing unnecessary shaking.

3. Use of equipment with a minimum number of parts.
4. Prompt refrigeration of blood, if it is to be stored.
5. Fasting, carefully selected, healthy donors.
6. Proper cleansing and prompt autoclaving of all material.

For this reason our technique is included:

1. Disconnect all glass from rubber parts upon return of set.
2. Treatment of rubber parts.
   (a) Rinse with tap water *at high pressure* for 5 minutes.
   (b) Stretch tubing; rinse with sterile distilled water.
   (c) Boil in sterile distilled water for 10 minutes.
   (d) Rinse thoroughly with distilled water.
   (e) Assemble and autoclave within two hours.
3. Treatment of used glass parts.
   (a) Small Parts.
      1. Wash clean with tap water.
      2. Boil in sterile distilled water for ten minutes.
      3. Rinse thoroughly with sterile distilled water.
      4. Assemble and autoclave within two hours.
   (b) Glass Flasks.
      1. Wash flasks with brush dipped in soap solution.
      2. Rinse with cold tap water for three minutes.
      3. Rinse with hot tap water for three minutes.
      4. Rinse thoroughly with sterile distilled water for six changes.
      5. Assemble and autoclave within two hours.
4. All equipment is autoclaved at twenty pounds for twenty minutes.
5. Preparation of new rubber tubing.
   (a) Rinse with tap water at high pressure for two hours.
   (b) Immerse in 5 per cent sodium carbonate and autoclave for thirty minutes at twenty pounds pressure.
   (c) Rinse with tap water at high pressure for three hours.
   (d) Drain well.
6. When it is impossible to clean glassware as above outlined, sulphuric-dichromate cleaning solution is used. Rinse with tap water and sterile distilled water as above.

In a series of 920 transfusions given by the entire resident staff in this hospital, there have been no serious reactions and no deaths. Following 0.9 per cent of transfusions, mild reactions

occurred. Most of the blood in this series has been refrigerated; in some cases, up to 21 days. We now have reason to believe that seven days is the maximum safe period of storage for citrated whole blood. Therefore, it is now our practice to store whole blood for seven days only, after which time the plasma is removed and the cells discarded. Plasma will suffice for all occasions where cellular replacement is not a prime requisite.

SUMMARY

1. Equipment for transfusing citrated blood is described, which is durable, inexpensive and practical.

2. The following points were found to be significant in the reduction of the number of transfusion reactions: (a) Avoid heating the blood. (b) Avoid unnecessary handling of the blood. (c) Avoid use of harsh cleansing materials. (d) Prompt refrigeration at 4°C. (e) The use of practical equipment. (f) Autoclave equipment within two hours from beginning of the cleansing procedures.

3. In 0.9 per cent of 920 transfusions mild reactions occurred.

ANNOTATIONS MINOR CONTRIBUTIONS QUERIES

A SIMPLIFIED METHOD FOR THE DETERMINATION OF BLOOD UREA

Dr. Fabry L. Hawks, co-author of the above paper which appeared in the September 1938 issue of the Supplement, writes that the following methods for preparing the standard nitrogen solution and Nessler's working solution are simpler and easier than those described at that time.

In preparing standard ammonium sulphate this may be prepared from Folin's solution of standard ammonium sulphate 1 cc. equalling 0.1 cc. nitrogen, by adding to 50 cc. of distilled water into a 250 cc. volumetric flask 37.5 cc. and diluting to volume with distilled water.

In preparing working Nessler's solution dilute 30 cc. of stock Nessler's solution with an equal amount of distilled water. To 30 cc. of the diluted Nessler's add 70 cc. of a freshly prepared 5 per cent sodium hydroxide solution (Baker's or Merck's Pellets). The stock Nessler's solution is the one used as in making working Nessler's as for non-protein nitrogen determination, Folin's nitrogen determination etc. The other stock Nessler's need not be made unless one desires.

A NEW STAIN FOR OPSONOCYTOPHAGIC TESTS

In our hands slides for opsonocytophagic tests for brucellosis stained with Hastings stain have in many instances offered great difficulty in differentiation between cytoplasmic granules and bacteria. In a search for a better stain, we have tried Loeffler's methylene blue, Manson's borax methylene blue as used in basophilic aggregation stains, Wright's stain and hematoxylin and eosin. None of these seemed to us to be much better than Hasting's stain. Recent work on solvents for Romanowsky stains suggested the possibility of dissolving powdered Wright's stain in acetone. After much experimentation with concentration of the dye and the time of staining, the following formula was adopted.

Wright's stain	0.6	grams
Acetone, C. P	95	cc.
Glycerine	5	cc.

This stock staining solution should age about 7 days before use. The air dried slides are covered with the stain for 3 minutes. Distilled water is added until there is definite metallic scum. Staining is then continued for an additional 7 minutes. The slide is then washed with water, dried immediately and is ready for examination.

The red cells stain a lighter pink color than with Wright's stain. The nuclei of the white cells and the bacteria stain a deep blue and the cytoplasm of the polymorphonuclears is a clear, very light pink. No cytoplasmic granules are seen.

The occasional failure to stain properly is due either to improper dilution or to a delay in drying following the final wash with water. *H. E. Cope and Helen Jaffe, Owen Clinical Laboratory, Detroit, Michigan.*

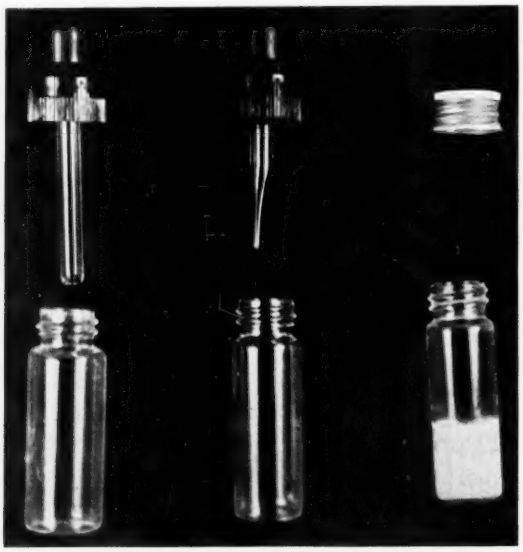
A SUGGESTED CONTAINER FOR ASEPTIC COLLECTION OF CHANCRE FLUID, PUS, ETC.

The container is made from a standard medicinal dropper vial $\frac{1}{8}$ ounce size. The dropper tip is pulled out to a capillary tip by heating in the flame. (It is best to stick another piece of glass tubing on the end of the applicator so that one can heat the dropper stem and pull it out without burning the fingers.) After pulling out the stem to a capillary tip, the tip is cut off to a length which allows it to be placed inside the vial, coming to within about $\frac{1}{4}$ inch of the bottom. Ordinarily, these vials are used without further preparation except autoclaving. If it is desired to send material which one wishes to prevent from drying, another vial is filled about one-third full of the mixture of paraffin and vaseline, the consistency of which should remain solid at warm room temperatures.

Material can be collected from wounds, sores, etc., drawn up into the pipette and the tip of the pipette pushed down into the paraffin-vaseline mixture and

the tip screwed on. In this condition, the liquid part of the specimen will not evaporate to any appreciable extent for several weeks. A further advantage of the container is that after filling with infective material, the lid can be tightened down and the whole vial immersed into disinfectant and thus protect the worker from exposure to infected material.

The cost of these vials is approximately 5 cents apiece. Type of vial used was "Glasco Dropper Vial," #7580. *G. E. Culbertson, M.D. Central Laboratories, Indiana University Medical Center.*

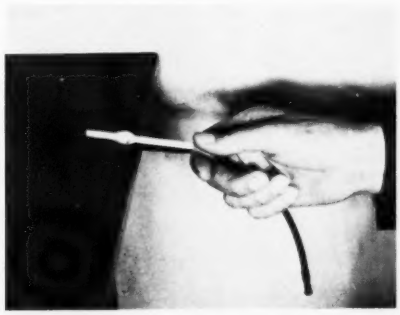


A SIMPLE AND EFFICIENT METHOD FOR CLOSING BLOOD PIPETTES

The method described below has been used for the past four years and has proven very satisfactory. It therefore is passed on with the hope that other laboratories will find it useful.

An approximate one-inch length of ordinary red or white rubber tubing of medium thickness and of a size which fits snugly over the pipette tubing is slipped over the latter. To most easily do this a small hemostat is pushed through the short piece of tubing, the pipette tubing grasped, stretched, and pulled through. The collar of tubing is adjusted so that when the pipette is turned back on its attached tubing and the tip of the pipette inserted under the collar the pipette tubing is on moderate stretch and tightly seals the large end of the pipette.

This arrangement facilitates shaking the pipette as it can be easily bounded on the table as shown in the photograph. This movement causes a marked excursion of the bead and mixes the contents quickly.



Where it is desired to mark the pipettes, numbers may be written in India ink on the collars of tubing.

A word of caution: As in any similar method of closure the blood and diluting fluid must be well mixed before the tubing is stretched over the large end of the pipette as a small amount of fluid will necessarily be forced from the pipette in this act. *E. E. Myers, M.D., Philippi, W. Va.*